Appendix B2

Protocol for the COS Cell Binding Assay

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COS CELL BINDING ASSAY

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1. Day 1- Monday

Plate 400,000 COS-1 cells/well of 6 well plate in 3 ml 10% bovine calf serum, DMEM-H/20 mM Hepes, glutamine, pen/strep (use stock of 2 M Hepes, pH 7.2, sterile filter)

(200,000 cells/12 well plate with 2 ml media for Scatchard analysis)

2. Day 2, prepare DNA

0.95 ml 1.08x TBS/well
2 µg AR DNA for 6 well comp binding (0.1-3 µg AR DNA for 12 well, 3 µg for GAL/VP vectors)
0.11 ml DEAE-dextran
(250 mg/50 ml, autoclaved water, sterile filter made fresh)

Aspirate media, add 1 ml DNA solution, incubate 30 min at 37˚C, aspirate media
Add 3 ml/6 well of chloroquine-media (2 ml/12 well)
Prepare 5 mg/ml chloroquine in dH_2O fresh, sterile filter, add 1 ml of 5 mg/ml chloroquine to 100 ml 10% BCS/DMEM-H, 20 mM Hepes media
Incubate 3 h at 37˚C, aspirate media
Glycerol shock 4 min at RT with 1 ml/6 well (or 12 well) of 15% glycerol in 10% BCS/DMEM-H
Aspirate, wash carefully 1X with 3 ml 1xTBS/6 well (2 ml/12 well)
Add 3 ml 10% BCS DMEM-H, incubate overnight in incubator

3. Day 3, leave in 10% serum containing media until next day

4. Day 4, aspirate (don't wash), set up tubes for binding assay:

Use 600 µl/6 well of 5 nM [^{3}H]R1881 labeling solution in serum free/phenol red free with or without 100 fold excess unlabeled R1881 for nonspecific binding control (400 µl/12 well)

For calculations, prepare 0.625 ml/well for all h and h+c wells in serum-free, phenol red-free media

To make h + c, # h+c wells x 0.625 ml, take this volume from 5 nM hot solution, add cold R1881 so final is 100 fold higher (500 nM) unlabeled R1881 with 5 nM [^{3}H]R1881

Incubate 2 hr at 37˚C (for Scatchard in 12 well, after 2 h labeling, take 100 µl for free counts)

For ligand dissociation experiment:

Add 10,000 fold excess of cold R1881 (50 µM final) in 0.1 ml serum free media (350 µM, 7X stock)

Amount to prepare: 100 µl x total # wells + 0.5 ml extra

Spread plates out in incubator, start timer, incubate at 37˚C for times indicated
Remove at indicated time, aspirate using radioactive flask; wash carefully 1X with 3 ml PBS
Aspirate to dry, harvest in 500 µl 1X sample buffer (2% SDS, 10% glycerol, 10 mM Tris, pH 6.8) for 6 or 12 well, add 4 ml scintillation fluid and count

	500 ml	final conc	4 liters
2X TBS:	8.18 g NaCl	280 mM NaCl	65.44 gr NaCl
	0.23 g KCl	6 mM KCl	1.84 g KCl
	0.147 g $CaCl_2$-$2H_2O$	2 mM $CaCl_2$	1.18 g $CaCl_2$-$2H_2O$
	0.1 g $MgCl_2$-$6H_2O$	1 mM $MgCl_2$	0.8 g $MgCl_2$-$6H_2O$
	0.128 g NaH_2PO_4-H_2O	1.8 mM NaH_2PO_4	1.02 NaH_2PO_4-H_2O
	3.03 g Tris	50 mM Tris pH 7.4	24.24 g Tris

pH to 7.4

	500 ml	4 liters	final	MW
1.08X TBS:	4.42 g NaCl	35.34 g NaCl	51.2 mM NaCl	58.44
	0.121 g KCl	1.0 g KCl	3.24 mM KCl	74.56
	0.08 g $CaCl_2$-$2H_20$	0.64 g $CaCl_2$-$2H_20$	1.08 mM $CaCl_2$	147.02
	0.055 g $MgCl_2$-$6H_20$	0.439 g $MgCl_2$-$6H_20$	0.54 mM $MgCl_2$	203.3
	0.067 g NaH_2PO_4-H_20	0.54 g NaH_2PO_4-H_20	0.972 mM NaH_2PO_4	137.99
	1.636 g Tris	13.09 g Tris	27 mM Tris pH 7.4	121.14

pH to 7.4
or 270 ml 2XTBS + 230 ml H_20 = 1.08xTBS